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Toxicological evaluation of sodium perfluorohexanoate

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ABSTRACT

Sodium perfluorohexanoate [NaPFHx, $F(CF_2)_5CO_2Na$, CAS#2923-26-4] was evaluated in acute, 90-day subchronic, one-generation reproduction, developmental and *in vitro* genetic toxicity studies. In the subchronic/one-generation reproduction study, four groups of young adult male and female Crl:CD(SD) rats were administered NaPFHx daily for approximately 90 days by gavage at dosages of 0, 20, 100, or 500 mg/kg. Selected groups of rats were evaluated after 1- and 3-month recovery periods. Rats selected for reproductive evaluations were dosed for approximately 70 days prior to cohabitation, through gestation and lactation, for a total of about 4 months. The subchronic toxicity no observed adverse effect level (NOAEL) was 20 mg/(kg day), based on nasal lesions observed at 100 and 500 mg/(kg day). No effects were observed for neurobehavioral endpoints. NaPFHx was a moderate inducer of hepatic peroxisomal β-oxidation with a no observed effect level (NOEL) of 20 (male rats) and 100 mg/(kg day) (female rats). Elevated hepatic β-oxidation levels were observed following 1-month recovery in male and female rats at 500 mg/(kg day). No NaPFHx-related effects were observed on any reproductive parameters. The P_1 adult rat NOAEL was 20 mg/(kg day), based on reduced body weight parameters, whereas the NOAEL for reproductive toxicity was 100 mg/(kg day), based on effects limited to reduced F_1 pup weights. In the developmental study, female rats were dosed via gavage on gestation day (GD) 6–20 with the same doses of NaPFHx administered in the subchronic study. The maternal and developmental toxicity NOAEL was 100 mg/(kg day), based on maternal and fetal body weight effects at 500 mg/(kg day). NaPFHx is therefore concluded not to present a reproductive or developmental hazard. NaPFHx genotoxicity studies showed no mutations in the bacterial reverse mutation (Ames) assay or chromosome aberrations in human lymphocytes treated with NaPFHx *in vitro*. The lowest NOAEL from all of the studies was 20 mg/(kg day) in the subchronic study based on nasal lesions. Benchmark doses (BMDL10) for nasal lesions were 13 and 21 mg/(kg day) for male and female rats, respectively. The relevance of the nasal lesions to humans is not known.

1. Introduction

Perfluoroalkyl acids (PFAAs), such as perfluoroalkyl sulfonates (PFAS) and perfluorocarboxylic acids (PFCAs), have been found widely in the environment (Houde et al., 2006). The predominant acids observed in the environment, including human blood, are perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA). PFOS, PFOA, and perfluorohexane sulfonate (PFHxS) have very long half-lives in humans (Olsen et al., 2007), and are said to be biopersistent, which is a measure of the tendency of a chemical substance to stay in a living system for an extended period of time, as typically measured by half-life or clearance time. The sources of PFAS (3M Company, 1999) and PFCAs (Armitage et al., 2006; Prevedouros et al., 2006) containing four or more fluorinated carbons have been described. Toxicological studies of PFAS, including PFOS, PFHxS, and perfluorobutane sulfonate (PFBS) (OECD Environmental Directorate, 2002; Seacat et al., 2003; Lau et al., 2007; Butenhoff et al., 2009; Lieder et al., 2009), and PFCAs, including PFOA and perfluorobutanoic acid (PFBA) (Kennedy et al., 2004; Das et al., 2008; J. Butenhoff, personal communication, 2009), have been recently reported. In addition, the major global manufacturer of PFAS and PFOA ceased manufacture and industry has committed to reduce product content and emissions of PFOA, higher homologues and related precursors and work toward the elimination of PFOA and higher PFCA homologues and related precursors [United States Environmental Protection Agency (USEPA), 2006].

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PFCAs and PFAS with five or fewer fluorinated carbons, such as PFBA, PFBS and perfluorohexanoic acid (PFHxA), have also been found in the environment (Falandysz et al., 2006; Lange et al., 2007; Hoelzer et al., 2008). In contrast to PFOS, PFHxS and PFOA, PFBS and PFBA have short half-lives in humans and monkeys (Lieder et al., 2006; Chang et al., 2008; Olsen et al., 2009). Moreover, a recent critical review concluded that PFCAs with seven or fewer fluorinated carbons are not bioaccumulative according to regulatory criteria (Conder et al., 2008). In a rainbow trout bioaccumulation study, perfluorohexanoate (PFHx) was not detected in fish tissue and it was concluded that PFHxA had negligible bioaccumulation potential (Bioaccumulation Factor < 0.1) (Martin et al., 2003a). In a rainbow trout bioconcentration study, perfluoroalkyl carboxylates with less than seven carbons had insignificant bioconcentration factors (Martin et al., 2003b).

The toxicology of PFOS and PFOA has been the subject of numerous reviews (OECD Environmental Directorate, 2002; Kennedy et al., 2004; Lau et al., 2007). Both compounds have shown reduced body weight and increased liver weight in repeated-dose studies. In various mutagenicity assays, both compounds have been reported to be negative (Griffith and Long, 1980; 3M Company, 1996; Health Canada, 2006). Effects on body and liver weight parameters were also observed in male rats following subchronic oral gavage to 10 mg/(kg day) PFHxS (Butenhoff et al., 2009). In contrast, no effects on body and liver weights were reported following 90-day dosing of up to 600 mg/(kg day) PFBS, although hematologic effects were reported in male rats (Lieder et al., 2009).

The liver response in male and female mice following five daily intraperitoneal doses of PFCAs of various chain lengths, in relation to induction of hepatomegaly, peroxisomal β-oxidation and microsomal 1-acylglycerophophocholine acyltransferase, has been reported (Kudo et al., 2006). In general, potency increased with increasing chain length. For example, the doses required to induce a 1.5-fold increase in relative liver weight were >150, 40, 2.5 and 2.5 mg/kg body weight/day for PFHxA, perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), and perfluorononanoic acid (PFNA), respectively. While many repeated dose toxicity studies have been published for PFOS, PFHxS, PFBS, PFOA and PFBA, published data for PFHxA has only recently become available (Chengalis et al., 2009a). Doses were similar to those used in the current study and, for the most part, similar endpoints were reported to be affected, e.g., body, liver and kidney weights; red blood cell parameters.

To achieve the goal of eliminating PFOA and higher PFCA homologues and related precursors (USEPA, 2006), global fluorotelomer manufacturers are creating new products with shorter fluorinated chain-length functionality, e.g., $F(CF_2)_6CH_2CH_2-$. A potential degradation product from these new products and their raw materials is PFHxA. A better understanding of PFHxA toxicology is therefore needed in order to assess its potential hazards. Because the pKa of PFHxA is less than 3, it exists in the environment principally as an anion, PFHx. The objective of this study, therefore, was to characterize the acute, repeated-dose subchronic, one-generation reproduction, developmental and genetic toxicity profile for sodium perfluorohexanoate (NaPFHx).

2. Materials and methods

Methods and materials used in this study have been described previously (Ladics et al., 2005; Mylchreest et al., 2005; Stadler et al., 2008). Where methods differ, the details are as follows.

2.1. Test substance and administration

NaPFHx was supplied by DuPont Chemical Solutions Enterprise, Wilmington, DE, as a white solid, 100% purity. Analysis indicated the test substance was stable throughout the course of the studies. NaPFHx was diluted in NANOpure® water and administered by oral gavage to achieve dosage levels of 0, 20, 100, or 500 mg/(kg day) for 90-day subchronic, one-generation reproduction, and developmental studies. Dose volumes were 5.0 mL/kg. Male and female control animals were similarly treated with deionized water at the same dose volume as used in the other groups.

2.2. Animals and animal husbandry

Crl:CD(SD) rats were obtained from Charles River Breeding Laboratories, Raleigh, NC, USA. Rats for the subchronic and reproduction studies were approximately 4 weeks old when received and 6–8 weeks old at study start. Time-mated females for the developmental toxicity study were either at 1, 2, or 3 days of gestation when received. Rats were housed and maintained in accordance with the principles described in the *Guide to Care and Use of Laboratory Animals* (National Research Council, 1996). Tap water and pelleted chow (Certified Rodent LabDiet® 5002: PMI® Nutrition International, LLC) were available *ad libitum* throughout the study.

2.3. Acute toxicity

A single dose of 175, 550, 1750, or 5000 mg/kg was administered to fasted female rats, which were observed for up to 14 days for clinical signs of toxicity, body weight effects, and mortality.

2.4. 90-Day subchronic toxicity study

Dosages were set at 20, 100, and 500 mg/(kg day), based upon rangefinder studies. Rats were divided into groups with 55 rats/sex in the 500 mg/(kg day) dose and control groups, and 45 rats/sex in the 100 mg/(kg day) and 20 mg/(kg day) dose groups (see Fig. 1). Of these, 10 rats/sex/dose were designated for evaluation of subchronic toxicity; the next 20 rats/sex/dose were designated for reproductive assessment; 10 rats/sex/dose (control and high-dose groups only) were designated for 30-day recovery assessment and 10 rats/sex/dose were designated for 90-day recovery assessment. An additional subgroup (5 rats/sex/dose) was received as a separate shipment and was added to each dose group approximately 84 days after study start, for evaluation of hepatic peroxisomal β-oxidation analysis following a 10-day exposure. This study conformed to OECD Guideline 408 (Repeated Dose 90-Day Oral Toxicity Study in Rodents).

Rats were weighed at regular intervals, cage site examinations were conducted daily, and detailed clinical observations were recorded weekly. Ophthalmological and neurobehavioral evaluations, including functional observations (i.e., grip strength, sensory motor function) and motor activity assessments, were conducted prior to study start and during the last week of dosing. Clinical pathology evaluations were conducted on day 45, day 92 (males) or 93 (females), and at 1 month of recovery. Blood for plasma fluoride analysis was collected from the *vena cava* at sacrifice. Urine fluoride was determined at the end of treatment and at the end of the 1-month recovery period. Gross examinations were performed at sacrifice. Selected tissues were weighed and/or processed for microscopic examination, as previously described (Ladics et al., 2005).

2.5. Hepatic peroxisomal β-oxidation evaluation

After 10 or approximately 90 days of administration, or following a 30-day recovery period (control and high dose only), β-oxidation activity from liver microsomes was measured in 5 overnight-fasted rats from each group. Livers were removed and weighed, and hepatic peroxisomes were prepared and peroxisomal β-oxidation activity determined as described by Biegel et al. (2001).

2.6. One-generation reproduction study

P1 female rats selected for reproductive evaluations were dosed by oral gavage for approximately 70 days prior to cohabitation, through gestation and lactation for a total of approximately 126 days. P1 male rats in this subset were dosed for a total of approximately 110 days. F1 rats were not dosed. Clinical observations, body weight, and food consumption were determined weekly throughout the study. Estrous cycle, sperm parameters, survival, and reproductive performance parameters were assessed. Litter examinations (number of live and dead, individual pup weights, clinical observations) were determined on day 4, and weekly during the lactation period. F1 offspring were given a gross pathological examination at weaning. A subset of F1 generation rats was maintained for 6 weeks after weaning to assess developmental landmarks. The subset was given a gross pathological examination and selected reproductive organs were weighed. This study was in alignment with OECD Guideline 415.

2.7. Developmental toxicity study

Twenty-two female rats per group were dosed once daily on days 6–20 of gestation according to OECD Guideline 414. In-life observations were recorded and rats sacrificed on gestation day (GD) 21. All dams underwent a gross pathological examination and the fetuses were removed from the uteri by Cesarean section. Fetuses were weighed and sexed and examined for morphological alterations. All fetuses were examined for external and skeletal alterations, and approximately 50% of the fetuses were examined for soft tissue and visceral head examinations.

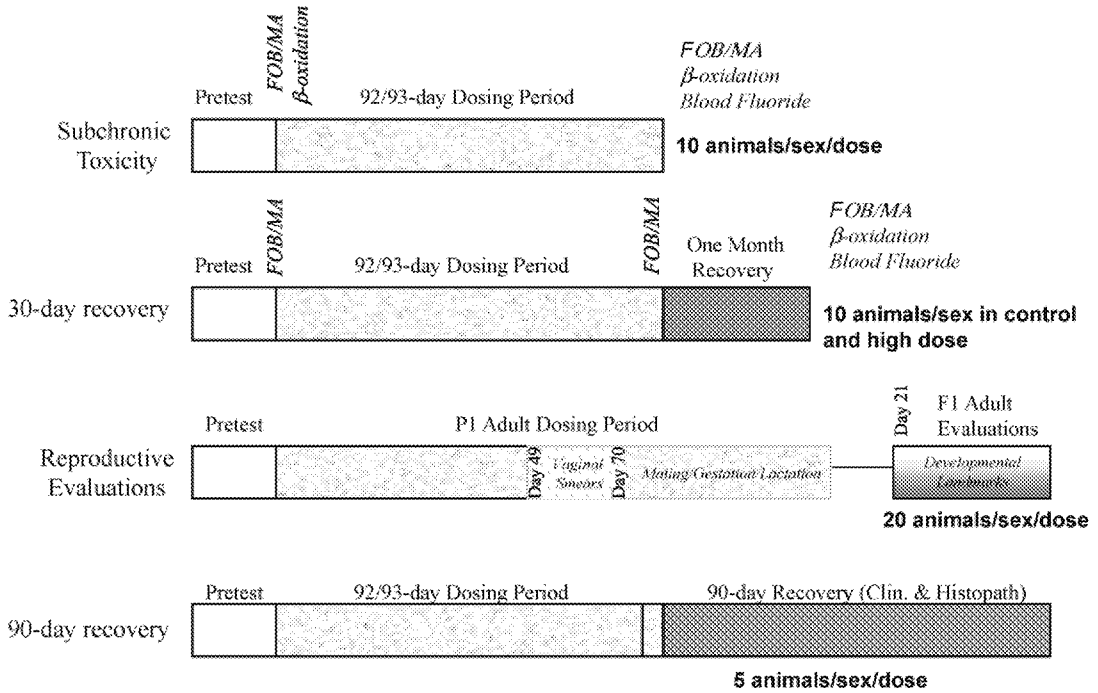

Fig. 1. Schematic overview of 90-day subchronic study with 30- and 90-day recovery groups and a one-generation reproduction study. FOB/MA = Functional Observation Battery/Motor Activity.

2.8. Genotoxicity testing

NaPFHx was evaluated for mutagenicity in the bacterial reverse mutation (Ames) assay using the plate incorporation method, according to OECD Guideline 471. *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* strain WP2*uvr*A were tested in the presence and absence of an exogenous metabolic activation system (Aroclor-induced rat liver S9). All tester strains and supplies, as well as liver S9 fraction prepared from male Sprague–Dawley rats induced with Aroclor 1254, and positive controls of benzo[a]pyrene, 4-nitroquinoline N-oxide, acridine mutagen ICR-191, sodium azide, 2-aminoanthracene, and 2-nitrofluorene, were purchased from Moltox Inc. (Boone, NC). The test was performed in two phases. The first phase was the toxicity-mutation test which established the dose range for the mutagenicity test. The second phase was the mutagenicity test which evaluated and confirmed the mutagenic potential of NaPFHx. NaPFHx was soluble in sterile water at 50 mg/mL, the highest concentration that was tested. The dose levels used were 333, 667, 1000, 3333, and 5000 μg/plate for all tester strains (TA98, TA100, TA1535, TA1537, and WP2*uvr*A) in the presence and absence of S9 metabolic activation.

The plate incorporation method was employed. NaPFHx was evaluated for its ability to induce structural and numerical chromosome aberrations *in vitro* using human peripheral blood lymphocytes (HPBL) in the absence and presence of an exogenous metabolic activation system (Aroclor-induced rat liver S9) according to OECD Guideline 473. Human blood was collected from a healthy volunteer donor. The positive controls, mitomycin C and cyclophosphamide (CP), were purchased from Sigma–Aldrich (St. Louis, MO). Cell culture media and buffers were obtained from Mediatech (Manassas, VA).

To establish a concentration range for the chromosome aberration assay, a preliminary toxicity test was conducted. NaPFHx was soluble in water at 38.6 mg/mL (100 mM). The cells were treated for 4 or 22 h in the non-activated test condition and for 4 h in the S9-activated test condition. All cells were harvested 22 h after treatment initiation. The maximum concentration tested in the preliminary toxicity assay based on the formula weight of NaPFHx was 10 mM (3860 μg/mL), the recommended limit dose for this test system (i.e., 5000 μg/mL or 10 mM, whichever is lower – OECD 473 testing guideline).

The cells were exposed to nine concentrations ranging from 5 to 3860 μg/mL, as well as vehicle controls. No visible precipitate was observed in the treatment medium at the beginning or end of the treatment period at any concentration tested. Based on the results from the toxicity test, the chromosome aberration assay was conducted with cytogenetic evaluations conducted at 2000, 3000, and 3860 μg/mL (10 mM) for the 4-h non-activated test condition and at 250, 500, and 1000 μg/mL (2.59 mM) for the 4-h activated and 22-h non-activated test conditions.

2.9. Statistical analyses

A variety of statistical tests were used, as appropriate, for each study. Preliminary tests were conducted for homogeneity of variance (Levene, 1960) and normality (Shapiro and Wilk, 1965). A one-way analysis of variance (Snedecor and Cochran, 1967) followed by Dunnett's test (Dunnett, 1964) were conducted if data were normally distributed and had homogeneity of variance. For data that did not show homogeneity of variances, a robust version of Dunnett's test (Dunnett, 1980) was used. Non-normally distributed data were analyzed using the non-parametric Kruskal–Wallis test (Kruskal and Wallis, 1952) followed by Dunn's test (Dunn, 1964). For all statistical analyses, significance was judged at $p < 0.05$. Comparisons were made between the vehicle control group and the dosed or positive control groups. Statistical methods applied in the subchronic, reproductive, and developmental toxicity studies were as follows. Monotonal endpoints were evaluated with Levene's test for homogeneity and Shapiro–Wilk test for normality. If the Shapiro–Wilk test was not significant, but Levene's test was significant, a one-way analysis of variance followed by Dunnett's test (Dunnett, 1980) was used. If the Shapiro–Wilk test was significant, Kruskal–Wallis test was followed by Dunn's test. No statistical evaluation was conducted to determine the significance of the nasal lesions.

3. Results

3.1. Acute

All rats dosed with 175 or 550 mg NaPFHx/kg survived, whereas one of four rats dosed with 1750 mg/kg died on the day of dosing. All three rats dosed with 5000 mg/kg were found dead on the day of dosing or the day after dosing. Clinical signs of systemic toxicity were observed in most rats and included abnormal gait, dehydration, high or low posture, clear oral or nasal discharge, wet or stained fur, salivation, ataxia, or lethargy.

3.2. 90-Day subchronic study

3.2.1. Mortality, clinical signs, and effects on body weight

No clinical signs of toxicity or mortality related to NaPFHx administration were observed in the rats during the course of the 90-day study at any dose level. Statistically significant decreases in mean body weight were observed in male rats in the high dose group (500 mg/(kg day)), beginning on study day 42 through study day 105 (2 weeks after ceasing dosing) (Fig. 2). Mean body weight on test day 91 was 90% of control and mean overall body weight gain (test days 0–91) was 81% of control. No NaPFHx-related effects on mean body weight or mean body weight gain were observed in

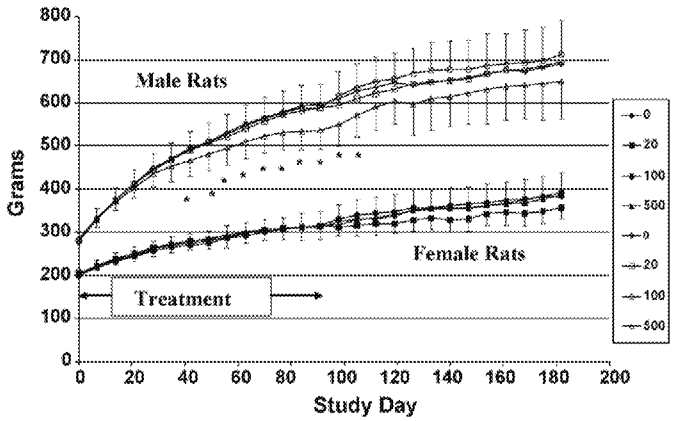

Fig. 2. Mean (±SD) body weights of male and female rats exposed to 0, 20, 100 or 500 mg NaPFHx/kg body weight/day for ~90 days. *$p < 0.05$, compared to vehicle control.

any female group after 90 days of dosing, or after 30 and 90 days of recovery for any group of male or female rats.

3.2.2. Food consumption

No adverse NaPFHx-related effects on mean food consumption were observed at any dose in males or females.

3.2.3. Ophthalmology

No NaPFHx-related ophthalmology findings were observed in any male or female group.

3.2.4. Functional observation battery and motor activity

There were no effects on any neurobehavioral parameters determined during functional observations (i.e., grip strength, sensory motor function) or motor activity assessments, at any dose in males or females.

3.2.5. Observations at necropsy

Macroscopic observations were normal for all rats sacrificed at the end of the study.

3.2.6. Clinical chemistries

There were no changes in clinical chemistry parameters indicative of NaPFHx-related organ toxicity (Table 1). Statistically significant differences from controls were observed for a number of parameters (e.g., aspartate aminotransferase, alanine aminotransferase, bilirubin, total protein), particularly in males dosed with 500 mg/kg, but these changes were considered non-adverse for a variety of reasons. Some of these reasons included low incidence, not occurring in a dose–response fashion, direction of change not associated with adversity, or the changes reflected adaptive responses following effects on the liver.

There were no statistically significant or NaPFHx-related changes in plasma fluoride concentrations at the end of the dosing period or after either recovery period. At day 92 or 93, the mean amounts of excreted fluoride (urine fluoride) were minimally increased in males and females administered 500 mg NaPFHx/(kg day) (Table 2). Urine fluoride was statistically significantly increased after 30 days of recovery in 500 mg/(kg day) females but not in males at test days 120 or 183 or in females at test day 184.

3.2.7. Hematology

Red blood cell count and hemoglobin and hematocrit values were mildly to moderately decreased at 500 mg/(kg day) in males (69%, 64%, and 69% of controls, respectively) and females (82%, 85%, and 87% of control, respectively) (Table 3). Changes in red blood cell indices were associated with increases in reticulocytes for 500 mg/(kg day) males and/or females and were indicative of an erythroid regenerative response. Increases in platelet counts in male and female rats were observed after 500 mg/(kg day) treatment. In males, increases in neutrophil counts were observed at all doses, but not in a dose–response fashion.

Results of hematology determinations performed after a 30- or 90-day treatment-free recovery period indicated recovery of treatment-related hematology changes at both recovery time intervals. No changes in coagulation, clinical chemistry or urinalysis parameters indicative of treatment-related overt organ toxicity were observed (data not shown).

3.2.8. Hepatic peroxisomal β-oxidation analysis

NaPFHx was evaluated for its ability to alter hepatic peroxisomal β-oxidation activity in male and female rats following 10 or approximately 90 days, and following a 30-day recovery period. NaPFHx induced hepatic peroxisomal β-oxidation activity in male rats at doses of 100 and 500 mg/(kg day), and in female rats at a dose of 500 mg/(kg day) at the 10-day and/or 90-day time points, with male rats induced to a greater extent than female rats at both time points (Fig. 3). The increases in hepatic β-oxidation persisted through the 30-day recovery time point, with a similar response in male and female rats at 500 mg/(kg day).

3.2.9. Organ to body weight data

Following 90 days of dosing, effects on organ weights were present in the liver and kidney of males (Table 4) and females (Table 5). Liver weight parameters were increased in male rats dosed with 100 and 500 mg/(kg day) and in female rats dosed with 500 mg/(kg day) (Fig. 4). Following both 30 and 90 days of recovery, liver weight effects in 500 mg/(kg day) males and females showed some, but not complete, recovery. The slight liver weight increase observed in 100 mg/(kg day) males at the end of dosing was reversible following 90 days of recovery.

Kidney weight parameters were minimally increased (111–117% of control) in male and female rats dosed with 100 and 500 mg/(kg day) (Fig. 5). Following 30 days of recovery, kidney weight effects in males showed some recovery in the 500 mg/(kg day) group.

Thyroid weight parameters had statistically significant increases in female rats in the 500 mg/(kg day) 30-day recovery group (data not shown). Similar changes were not seen in female rats at the end of the dosing period, after 90 days recovery, or in male rats at any time point.

A number of statistically significant organ weight changes occurred in male rats in the 500 mg/(kg day) group in a pattern consistent with effects occurring secondary to decrements in body weights (Table 3). For organ weights that are generally sensitive to body weight effects (spleen, thymus), organ changes were characterized by decreases in absolute weight with minimal or no changes in organ weight relative to body weight. For organs relatively insensitive to decrements in body weight (brain, testes), weight changes were characterized by increases in organ weights relative to body weight with minimal or no changes in absolute organ weight.

3.2.10. Histopathology

NaPFHx-related microscopic findings were present in the nose, liver, and thyroid gland. These changes were present in the nose of male and female rats administered 100 or 500 mg NaPFHx/(kg day). Incidences of these changes are summarized in Table 6. Minimal to mild degeneration/atrophy of olfactory epithelium was present in male and female rats in the 100 and 500 mg/(kg day) groups sacrificed at the end of the dosing period. Other nasal changes present at the end of the dosing period occurred primarily in

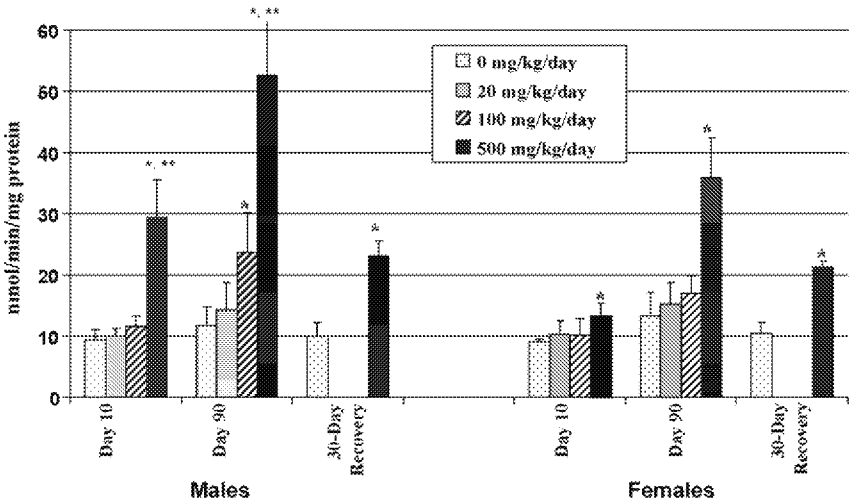

Fig. 3. Peroxisomal β-oxidation in male and female rats. After 10 or approximately 90 days of administration of NaPFHx, or following a 30-day recovery period (control and high dose only), β-oxidation activity from liver microsomes was measured in 5 overnight-fasted rats from each group. *$p < 0.05$, compared to vehicle control. **$p < 0.01$, compared to female cohort.

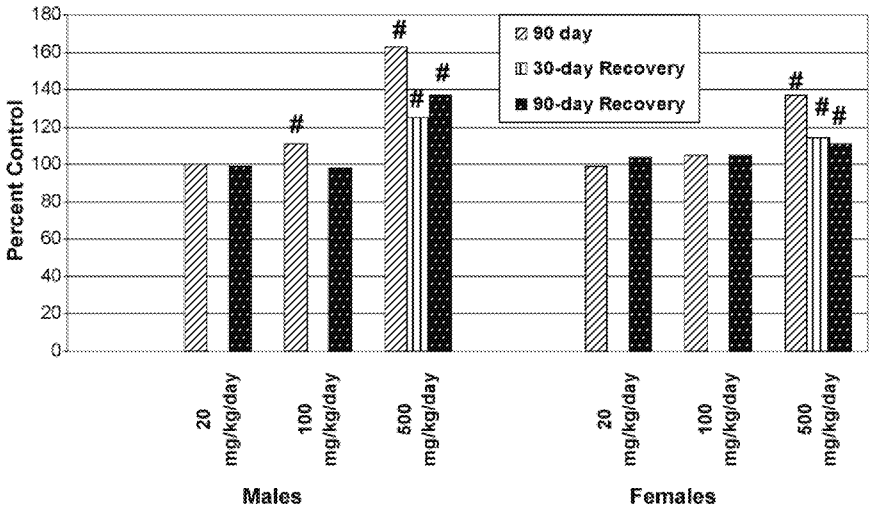

Fig. 4. NaPFHx effect on relative liver weights in male and female rats. After approximately 90 days of administration, or following a 30-day recovery period (control and high dose only), or a 90-day recovery period, mean liver weight to final body weight ratios were calculated and compared to control values. #NaPFHx-related effect.

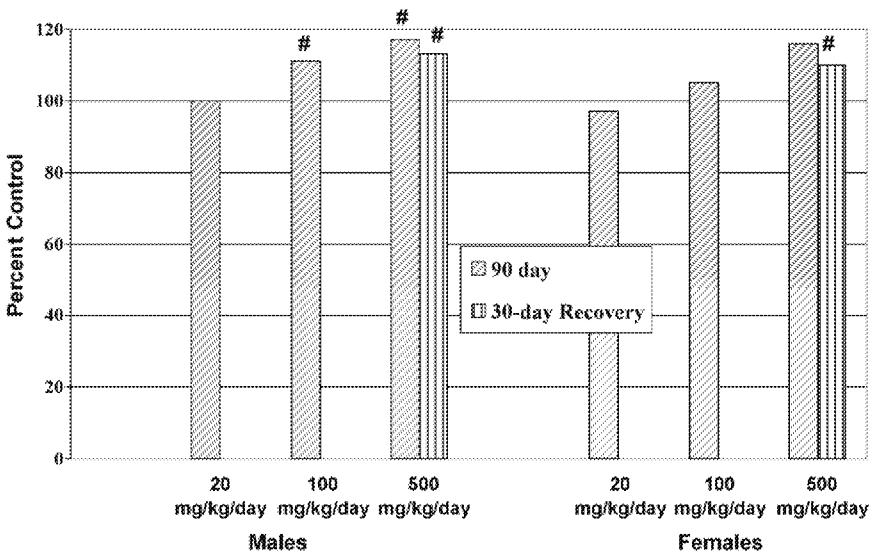

Fig. 5. NaPFHx effect on relative kidney weights in male and female rats. After approximately 90 days of administration, or following a 30-day recovery period (control and high dose only), mean kidney weight to final body weight ratios were calculated and compared to control values. #NaPFHx-related effect.

Table 1
Mean (±SD) clinical chemistry values in male and female rats.

	Male				Female			
Dosage (mg/(kg day))[a]	Control	20	100	500	Control	20	100	500
N	10	10	10	10	10	10	10	9[b]
AST (U/L)	69 ± 12	120 ± 94	86 ± 17[@,c]	96 ± 26[@,c]	171 ± 168	72 ± 19	95 ± 40	110 ± 82
ALT (U/L)	27 ± 5	63 ± 64[@,c]	39 ± 12[@,c]	42 ± 9[@,c]	69 ± 48	37 ± 10	52 ± 36	66 ± 60
SDH (U/L)	25 ± 6.1	32 ± 12	27 ± 4.5	18 ± 5.5[@,d]	29 ± 18	18 ± 4.4	21 ± 6.7	30 ± 21
ALKP (U/L)	81 ± 13	95 ± 26	97 ± 20	211 ± 89[@,d]	51 ± 19	43 ± 12	63 ± 18	42 ± 17
BILI (mg/dL)	0.12 ± 0.01	0.13 ± 0.02	0.10 ± 0.02[*,c]	0.09 ± 0.01[*,e]	0.17 ± 0.01	0.16 ± 0.02	0.14 ± 0.01[*,c]	0.09 ± 0.01[*,e]
BUN (mg/dL)	13 ± 1	13 ± 1	15 ± 1	16 ± 2[@,e]	16 ± 2	16 ± 2	16 ± 2	14 ± 3
CREAT (mg/dL)	0.39 ± 0.03	0.38 ± 0.04	0.38 ± 0.04	0.34 ± 0.03[*,e]	0.43 ± 0.03	0.43 ± 0.05	0.43 ± 0.04	0.37 ± 0.04[*,e]
CHOL (mg/dL)	62 ± 19	50 ± 15	40 ± 11[*,e]	55 ± 12[e]	93 ± 23	90 ± 27	78 ± 9	88 ± 16
TRIG (mg/dL)	49 ± 23	9 ± 21	43 ± 12	55 ± 15	47 ± 10	64 ± 70	40 ± 10	47 ± 13
GLUC (mg/dL)	104 ± 10	97 ± 15	105 ± 24	102 ± 7	104 ± 15	100 ± 9	107 ± 27	107 ± 8
TP (g/dL)	6.5 ± 0.4	6.5 ± 0.3	6.1 ± 0.3[*,c]	5.6 ± 0.4[*,c]	7.8 ± 0.7	7.7 ± 0.6	7.7 ± 0.4	7.6 ± 0.5
ALB (g/dL)	3.3 ± 0.2	3.3 ± 0.1	3.3 ± 0.1	3.3 ± 0.2	4.0 ± 0.4	3.9 ± 0.4	4.0 ± 0.1	4.2 ± 0.2
GLOB (g/dL)	3.2 ± 0.3	3.2 ± 0.2	2.8 ± 0.2[*,c]	2.3 ± 0.2[*,c]	3.8 ± 0.4	3.8 ± 0.3	3.7 ± 0.3	3.4 ± 0.4
CALC (mg/dL)	10.7 ± 0.3	10.5 ± 0.4	10.3 ± 0.3	10.2 ± 0.4[*,d]	11.6 ± 0.5	11.8 ± 0.5	11.6 ± 0.4	11.9 ± 0.4
PHOS (mg/dL)	7.6 ± 0.6	7.9 ± 0.8	8.2 ± 0.6	8.2 ± 0.6	5.8 ± 0.7	6.0 ± 0.6	5.9 ± 0.6	6.2 ± 0.4
NA (mmol/L)	149 ± 1.5	149 ± 1.4	149 ± 0.9	147.0 ± 1.8[*,d]	145 ± 2.0	146 ± 2.1	145 ± 1.3	143 ± 2.5
K (mmol/L)	6.0 ± 0.28	6.4 ± 0.48	6.3 ± 0.46	7 ± 0.72[*,d]	5.7 ± 0.25	5.7 ± 0.35	5.6 ± 0.39	5.4 ± 0.33
CL (mmol/L)	101 ± 2.1	101 ± 1.8	103 ± 1.4[*,c]	104 ± 3.3[*,d]	101 ± 2.9	100 ± 1.3	101 ± 1.9	101 ± 3.2
Plasma Fl (mg/μL)	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1

[a] Dosage occurred on days 1–92 (males) and 1–93 (females) of study.
[b] Excludes values for a rat found dead on day 5 of study.
[c] Parameter no longer statistically significantly different from control rats 3 months after cessation of dosing (*N* = 10 rats/group).
[d] Parameter no longer statistically significantly different from control rats 1 month after cessation of dosing (*N* = 10 rats/group).
[e] Parameter remained statistically significantly decreased from control rats 3 months after cessation of dosing (*N* = 10 rats/group).
[*] Statistically significant difference from control at $p < 0.05$ by Dunnett/Tamhane–Dunnett test.
[@] Statistically significant difference from control at $p < 0.05$ by Dunn's test.

the 500 mg/(kg day) groups in regions associated with olfactory degeneration/atrophy. These included low incidences of adhesions between adjacent ethmoturbinates or between ethmoturbinates and the septum. Also at the high dose, minimal respiratory metaplasia occurred. Following both the 30- and 90-day recovery periods, olfactory lesions were mostly reversible.

Following the 90-day dosing period, minimal hepatocellular hypertrophy was present in male rats administered 100 or 500 mg/(kg day) and in female rats administered 500 mg/(kg day). Following 30 days of recovery, incidences of minimal hepatocellular hypertrophy in 500 mg/(kg day) males and females were similar to that observed at the end of dosing. Following 90 days of recovery, minimal hepatocellular hypertrophy was limited to the 500 mg/(kg day) male rats.

Minimal hypertrophy of thyroid follicular epithelium was present in male and female rats in the 500 mg/(kg day) dose group and in a single male rat in the 100 mg/(kg day) dose group. Following 30 days of recovery, minimal follicular cell hypertrophy was present in the 500 mg/(kg day) groups, with incidences similar to that seen at the end of dosing. After 90 days of recovery, changes were limited to equivocal hypertrophy in 2 of 10 males in the 500 mg/(kg day) group. Changes occurring secondary to effects on red blood cells were observed in bone marrow (erythroid hypertrophy) and spleen (minimal to mild extramedullary hematopoiesis) in 500 mg/(kg day) males and females at the end of the dosing period but were not observed following 30 days of recovery.

3.3. Reproductive toxicity

3.3.1. Mortality, clinical signs and effects on body weight

There was no NaPFHx-related mortality in parental males or females at any dose tested. Clinical signs of toxicity included stained

Table 2
Mean (±SD) urinalysis values in male and female rats.

	Male				Female			
Dosage (mg/(kg day))[a]	Control	20	100	500	Control	20	100	500
N	10	10	10	10	10	10	10	9[b]
Volume (mL)	7.2 ± 3.4	8.3 ± 3.7	8.8 ± 3.1	22.1 ± 10.5[@,c]	6.1 ± 2.6	7.3 ± 3.1	6.9 ± 2.8	11.4 ± 5.9[*,d]
Osmolality (mOsm/kg)	1512 ± 492	1252 ± 382	1396 ± 676	797 ± 250[@,c]	1255 ± 461	1107 ± 298	1176 ± 404	889 ± 414
pH	6.7 ± 0.2	6.7 ± 0.3	6.7 ± 10.2	6.6 ± 0.2	6.6 ± 0.3	6.8 ± 0.4	6.9 ± 0.3	6.8 ± 0.4
Urobilinogen (Eu/dL)	0.4 ± 0.3	0.2 ± 0.0	0.3 ± 0.3	0.2 ± 0.0	0.2 ± 0.0[e]	0.2 ± 0.0[e]	0.2 ± 0.0[e]	0.2 ± 0.0[e]
Protein (mg/dL)	59 ± 23	30 ± 11	34 ± 18	7 ± 2[@,f]	22 ± 25	10 ± 5	10 ± 5	7 ± 4[@,g]
Fluoride (μg)	4.5 ± 0.8	4.5 ± 0.7	5.2 ± 0.9	7.8 ± 1.9[*,c]	3.3 ± 0.8	3.8 ± 1.2	3.8 ± 1.0	5.4 ± 1.6[*,f]

[a] Dosage occurred on days 1–92 (males) and 1–93 (females) of study. One-month recovery groups (control and 500 mg/(kg day)) and 3-month recovery groups (control, 20, 100, and 500 mg/(kg day)) were also evaluated (*N* = 10/group).
[b] Excludes values for a rat found dead on day 5 of the study.
[c] Parameter no longer statistically significantly different from control rats 1 month after cessation of dosing (*N* = 10 rats/group).
[d] Parameter statistically significantly different from control rats 1 month after cessation of dosing.
[e] Due to lack of variability among group means, statistical analyses were unable to be performed.
[f] Parameter no longer statistically significantly different from control rats 3 months after cessation of dosing (*N* = 10 rats/group).
[g] Parameter statistically significantly different from control rats 3 months after cessation of dosing.
[*] Significantly different from vehicle control by Dunnett/Tamhane–Dunnett test ($p < 0.05$).
[@] Significantly different from vehicle control by Dunn's test ($p < 0.05$).

Table 3
Mean (±SD) hematology values in male and female rats.

	Male				Female			
Dosage (mg/(kg day))[a]	Control	20	100	500	Control	20	100	500
N	10	10	10	10	10	10	10	9[b]
RBC ($\times 10^6$ μL^{-1})	8.89 ± 0.36	8.95 ± 0.34	8.46 ± 0.41	6.09 ± 1.27^{*,c}	8.34 ± 0.43	8.53 ± 0.52	8.32 ± 0.27	6.85 ± 0.63^{*,d}
HGB (g/dL)	15.4 ± 0.5	15.5 ± 0.41	4.5 ± 0.7	9.9 ± 2.8$^{@,d}$	15.6 ± 0.7	15.8 ± 0.8	15.6 ± 0.4	13.3 ± 0.9^{*,d}
HCT (%)	49.0 ± 1.4	49.2 ± 1.7	46.2 ± 1.8	34.0 ± 7.8$^{@,c}$	47.2 ± 2.3	47.7 ± 2.5	47.1 ± 1.4	41.2 ± 2.4^{*,d}
MCV (fL)	55.1 ± 1.7	55.1 ± 2.1	54.7 ± 2.4	55.8 ± 4.3	56.7 ± 1.0	56.0 ± 2.0	56.6 ± 1.7	60.3 ± 3.2^{*,d}
MCH (pg)	17.3 ± 0.6	17.3 ± 0.6	17.2 ± 0.8	16.1 ± 1.9	18.7 ± 0.3	18.6 ± 0.8	18.8 ± 0.6	19.5 ± 0.8^{*,d}
MCHC (g/dL)	31.4 ± 0.5	31.5 ± 0.5	31.5 ± 0.3	28.8 ± 1.8$^{@,c}$	33.0 ± 0.4	33.2 ± 0.4	33.1 ± 0.7	32.3 ± 0.6^{*,d}
RET ($\times 10^3$ μL^{-1})	174 ± 30.4	150 ± 24.0	167 ± 23.6	540 ± 212.0$^{@,c}$	142.9 ± 34.3	153.4 ± 36.8	161.8 ± 43.5	401.7 ± 158.9$^{@,c}$
PLAT ($\times 10^3$ mL^{-1})	1129 ± 98	1075 ± 91	1151 ± 133	1807 ± 253$^{@,d}$	1206 ± 247	1063 ± 187	1154 ± 156	1506 ± 322^{*,d}
WBC ($\times 10^3$ μL^{-1})	9.44 ± 0.90	11.1 ± 1.91	11.2 ± 3.29	10.3 ± 2.63	8.51 ± 2.21	8.23 ± 1.60	7.04 ± 1.12	9.11 ± 1.74
Neutrophils ($\times 10^3$ μL^{-1})	1.2 ± 0.27	1.9 ± 0.70*	1.6 ± 0.35^{*,c}	1.9 ± 0.65^{*,d}	1.1 ± 0.42	1.1 ± 0.45	0.97 ± 0.43	1.4 ± 0.71
Lymphocyte ($\times 10^3$ μL^{-1})	7.86 ± 0.75	8.72 ± 1.96	9.25 ± 3.05	8.19 ± 2.36	6.96 ± 1.77	6.70 ± 1.16	5.72 ± 1.03	7.31 ± 1.46
Monocytes ($\times 10^3$ μL^{-1})	0.21 ± 0.09	0.29 ± 0.14	0.18 ± 0.06	0.15 ± 0.10	0.24 ± 0.13	0.22 ± 0.09	0.17 ± 0.05	0.20 ± 0.08
Eosinophils ($\times 10^3$ μL^{-1})	0.12 ± 0.05	0.10 ± 0.03	0.10 ± 3.03	0.04 ± 0.03$^{@,c}$	0.11 ± 0.05	0.10 ± 0.04	0.09 ± 0.03	0.08 ± 0.04
Basophils ($\times 10^3$ μL^{-1})	0.04 ± 0.02	0.04 ± 0.01	0.04 ± 0.02	0.03 ± 0.04$^{@,d}$	0.08 ± 0.05	0.09 ± 0.05	0.07 ± 0.06	0.07 ± 0.05
PT (s)	14.9 ± 0.9	14.8 ± 0.5	15.0 ± 0.8	14.2 ± 0.9	14.8 ± 0.6	14.7 ± 0.6	14.7 ± 0.3	14.1 ± 0.3^{*,d}
APTT (s)	16.0 ± 1.6	14.2 ± 1.1*	14.5 ± 0.8*	12.7 ± 1.5^{*,d}	13.9 ± 0.8	13.7 ± 1.4	13.6 ± 1.7	13.9 ± 1.9

[a] Dosage occurred on days 1–92 (males) and 1–93 (females) of study. One-month recovery groups (control and 500 mg/(kg day)) and 3-month recovery groups (control, 20, 100, and 500 mg/(kg day)) were also evaluated (*N* = 10/group).
[b] Excludes values for a rat found dead on day 5 of the study.
[c] Parameter no longer statistically significantly different from control rats 3 months after cessation of dosing (*N* = 10 rats/group).
[d] Parameter no longer statistically significantly different from control rats 1 month after cessation of dosing (*N* = 10 rats/group).
[*] Significantly different from vehicle control by Dunnett/Tamhane–Dunnett test ($p < 0.05$).
[@] Significantly different from vehicle control by Dunn's test ($p < 0.05$).

skin/fur in males and females at 500 mg/(kg day). NaPFHx-related reductions in body weight parameters were observed in males at 100 and 500 mg/(kg day) (Table 7). At 100 and 500 mg/(kg day), overall body weight gain was reduced by 12 and 29%, respectively, as compared to the control group. There was a NaPFHx-related reduction in mean maternal body weight gain at 500 mg/(kg day) during the first but not subsequent weeks of gestation. During the lactation period, there were NaPFHx-related effects on body weight gains at 100 and 500 mg/(kg day). At both levels, expected reduced body weight gain was not observed throughout lactation. Animals at 100 and 500 mg/(kg day) gained an average of 20 and 25 g, respectively, throughout lactation, compared to an average gain of 5 g in the control group.

3.3.2. *Reproductive indices*

No NaPFHx-related effects were observed on mating, fertility, gestation length, number of implantation sites, estrous cyclicity, sperm parameters, litter size, sex ratio, pup clinical observations,

Table 4
Mean (±SD) organ weights (g) and organ weight to final body weight ratios (%) in male rats.

Organ	Measure	Dose group (mg/kg day)[a]			
		0 (*N* = 10)	20 (*N* = 10)	100 (*N* = 10)	500 (*N* = 10)
Adrenal glands	Weight (g)	0.071 ± 0.012	0.066 ± 0.008	0.064 ± 0.008	0.062 ± 0.012
	Ratio (%)	0.013 ± 0.001	0.012 ± 0.001	0.012 ± 0.002	0.012 ± 0.002
Brain	Weight (g)	2.19 ± 0.095	2.17 ± 0.108	2.17 ± 0.131	2.14 ± 0.096
	Ratio (%)	0.392 ± 0.028	0.387 ± 0.028	0.395 ± 0.020	0.427 ± 0.041*
Epididymides	Weight (g)	1.61 ± 0.122	1.64 ± 0.161	1.59 ± 0.099	1.56 ± 0.107
	Ratio (%)	0.287 ± 0.014	0.294 ± 0.039	0.290 ± 0.020	0.310 ± 0.024
Heart	Weight (g)	1.77 ± 0.19	1.71 ± 0.13	1.78 ± 0.17	1.70 ± 0.15
	Ratio (%)	0.315 ± 0.016	0.305 ± 0.023	0.325 ± 0.018	0.339 ± 0.034
Kidneys	Weight (g)	4.09 ± 0.57	4.07 ± 0.33	4.43 ± 0.77	4.25 ± 0.40
	Ratio (%)	0.728 ± 0.082	0.725 ± 0.046	0.806 ± 0.108	0.849 ± 0.105*
Liver	Weight (g)	15.09 ± 1.59	15.18 ± 2.07	16.49 ± 2.38	21.98 ± 2.35*
	Ratio (%)	2.687 ± 0.165	2.696 ± 0.255	2.994 ± 0.231	4.378 ± 0.485$^{@}$
Spleen	Weight (g)	0.86 ± 0.14	0.79 ± 0.11	0.80 ± 0.12	0.72 ± 0.09*
	Ratio (%)	0.153 ± 0.017	0.141 ± 0.022	0.145 ± 0.019	0.144 ± 0.021
Testes	Weight (g)	3.69 ± 0.29	3.74 ± 0.30	3.73 ± 0.33	3.85 ± 0.34
	Ratio (%)	0.659 ± 0.035	0.668 ± 0.063	0.681 ± 0.062	0.767 ± 0.080*
Thymus	Weight (g)	0.42 ± 0.13	0.37 ± 0.09	0.35 ± 0.11	0.29 ± 0.07*
	Ratio (%)	0.75 ± 0.023	0.067 ± 0.019	0.063 ± 0.018	0.057 ± 0.010
Thyroid gland	Weight (g)	0.025 ± 0.003	0.024 ± 0.004	0.022 ± 0.004	0.024 ± 0.003
	Ratio (%)	0.005 ± 0.000	0.004 ± 0.001	0.004 ± 0.001	0.005 ± 0.000

[a] Dosage occurred on days 1–92 of study.
[*] Statistically significant difference from control at $p < 0.05$ by Dunnett/Tamhane–Dunnett test.
[@] Statistically significant difference from control at $p < 0.05$ by Dunn's test.

Table 5
Mean (±SD) organ weights (g) and organ weight to final body weight ratios (%) in female rats.

Organ	Measure	Dose group (mg/kg day)[a]			
		0 (N = 10)	20 (N = 10)	100 (N = 10)	500 (N = 9[b])
Adrenal glands	Weight (g)	0.073 ± 0.011	0.079 ± 0.009	0.074 ± 0.011	0.075 ± 0.007
	Ratio (%)	0.025 ± 0.004	0.026 ± 0.005	0.26 ± 0.003	0.026 ± 0.002
Brain	Weight (g)	1.98 ± 0.090	1.95 ± 0.12	2.02 ± 0.10	2.00 ± 0.10
	Ratio (%)	0.671 ± 0.077	0.641 ± 0.071	0.708 ± 0.073	0.680 ± 0.026
Heart	Weight (g)	1.10 ± 0.09	1.10 ± 0.08	1.04 ± 0.11	1.08 ± 0.06
	Ratio (%)	0.369 ± 0.018	0.360 ± 0.032	0.365 ± 0.036	0.367 ± 0.021
Kidneys	Weight (g)	2.12 ± 0.34	2.12 ± 0.21	2.14 ± 0.21	2.42 ± 0.34
	Ratio (%)	0.709 ± 0.073	0.691 ± 0.074	0.747 ± 0.062	0.823 ± 0.086*
Liver	Weight (g)	7.98 ± 1.07	8.29 ± 1.05	8.04 ± 0.71	10.82 ± 1.05*
	Ratio (%)	2.682 ± 0.300	2.650 ± 0.158	2.807 ± 0.147	3.678 ± 0.176*
Ovaries	Weight (g)	0.133 ± 0.021	0.145 ± 0.025	0.142 ± 0.016	0.137 ± 0.023
	Ratio (%)	0.045 ± 0.008	0.047 ± 0.008	0.050 ± 0.006	0.047 ± 0.010
Spleen	Weight (g)	0.55 ± 0.09	0.51 ± 0.09	0.51 ± 0.05	0.53 ± 0.08
	Ratio (%)	0.185 ± 0.034	0.168 ± 0.026	0.177 ± 0.019	0.179 ± 0.020
Thymus	Weight (g)	0.29 ± 0.06	0.29 ± 0.11	0.25 ± 0.04	0.26 ± 0.05
	Ratio (%)	0.096 ± 0.016	0.092 ± 0.028	0.089 ± 0.014	0.088 ± 0.017
Thyroid gland	Weight (g)	0.20 ± 0.003	0.22 ± 0.003@	0.020 ± 0.003	0.022 ± 0.004
	Ratio (%)	0.007 ± 0.001	0.007 ± 0.001	0.007 ± 0.001	0.008 ± 0.001
Uterus	Weight (g)	0.67 ± 0.18	0.67 ± 0.28	0.91 ± 0.32	0.78 ± 0.15
	Ratio (%)	0.227 ± 0.070	0.221 ± 0.106	0.316 ± 0.098	0.263 ± 0.044

[a] Dosage occurred on days 1–93 of study.
[b] Excludes values for a rat which was found dead on day 5 of study.
* Statistically significant difference from control at $p < 0.05$ by Dunnett/Tamhane–Dunnett test.
@ Statistically significant difference from control at $p < 0.05$ by Dunn's test.

Table 6
Microscopic incidence of histopathological findings in male and female rats (N = 10 rats/group).

Tissue	Observation	Male				Female			
		0[a]	20[a]	100[a]	500[a]	0[a]	20[a]	100[a,b]	500[a]
Nasal cavity	Degeneration/atrophy, OE[c]								
	Main study	0	0	**4**	**7**	0	0	**5**	**4**
	30-Day recovery	0	–	–	0	0	–	–	0
	90-Day recovery	0	0	0	0	0	0	0	0
	Adhesions, turbinates								
	Main study	0	0	0	**3**	0	0	0	**3**
	30-Day recovery	0	–	–	**2**	0	–	–	**3**
	90-Day recovers	0	0	0	**5**	0	0	0	**2**
	Intraepithelial microcysts, OE								
	Main study	0	0	0	0	0	0	0	0
	30-Day recovery	0	–	–	**3**	0	–	–	**1**
	90-Day recovery	0	0	0	4	0	0	0	0
	Respiratory metaplasia								
	Main study	0	0	0	**4**	0	0	**1**	**7**
	30-Day recovery	0	–	–	**8**	0	–	–	**8**
	90-Day recovery	0	0	0	**8**	0	0	0	**4**
Liver	Hepatocellular hypertrophy								
	Main study	0	0	**4**	**10**	0	0	0	**5**
	30-Day recovery	0	–	–	**9**	0	–	–	**4**
	90-Day recovery	0	0	0	**6**	0	0	0	0
Thyroid	Thyroid hypertrophy								
	Main study	0	0	**1**	**2**	0	0	0	**4**
	30-Day recovery	0	–	–	**3**	0	–	–	**6**
	90-Day recovery	0	0	0	**2**	0	0	0	0

Bold values were interpreted to be NaPFHx-related effects. (–) Not evaluated.
[a] Dosage group (mg/(kg day)).
[b] In 100 mg/(kg day) females, the number examined was 11 and 9 for the main study and 90-day recovery groups, respectively.
[c] Olfactory epithelium.

Table 7
Mean (±SD) body weight gains (g) in P1 and F1 rats and body weights (g) of F1 pups.

Group		Day	Dosage (mg/(kg day))			
			Control	20	100	500
P1 males	Test	0–105	340 ± 44	329 ± 47	299 ± 43*	241 ± 40*
P1 females	Gestation	0–7	36 ± 10	38 ± 8	37 ± 10	25 ± 8*
		0–21	140 ± 25	145 ± 16	147 ± 23	134 ± 19
	Lactation	0–21	5.1 ± 26	7.4 ± 20	20 ± 15	25 ± 12*
F1 pups	Postnatal	0	7.1 ± 0.9	6.8 ± 0.6	6.3 ± 0.4	5.8 ± 0.4#
		7	18 ± 2.7	18 ± 2.2	17 ± 1.3	15 ± 1.4#
		14	36 ± 3.4	37 ± 3.0	34 ± 2.6	30.0 ± 2.5#
		21	60 ± 5.3	62 ± 5.0	57 ± 5.3	49 ± 4.1#
F1 males	Postweaning[a]	0–39	320 ± 25	327 ± 42	320 ± 27	321 ± 25
F1 females	Postweaning[a]	0–39	183 ± 21	178 ± 18	173 ± 21	183 ± 24

[a] Age of animals at postweaning day 0 = 21 days old.
* Statistically significant difference from control at $p < 0.05$ by Dunnett/Tamhane–Dunnett.
# Statistically significant difference from control at $p < 0.05$ by analysis of covariance and Dunnett–Hsu.

Table 8
Mean (±SD) maternal body weight gain (BWG) and fetal body weights (BW).

	Dosage (mg/(kg day))			
	Control	20	100	500
Maternal BWG (g)				
Gestation days 6–21	165 ± 18	167 ± 13	161 ± 17	134 ± 27@
Gestation days 6–21[a]	69 ± 10	69 ± 10	62 ± 11	51 ± 20@
Fetal BW (g)	5.8 ± 0.3	5.7 ± 0.3	5.8 ± 0.3	5.3 ± 0.6

[a] Total body weight gain (gestation days 6–21) minus products of conception on day 21.
@ Statistically significant difference from control at $p < 0.05$ by Dunn's test.

pup survival, or F_1 adult developmental landmarks at any dose tested. There were NaPFHx-related effects on mean pup weights at 500 mg/(kg day) (17–18% lower than control group) throughout lactation (Table 7). Overall body weight gain from test day 0 to 39 for F1 adults (postweaning) was comparable across dose levels for both sexes and no NaPFHx-related organ weight changes at any dose in F_1 adult males or females nor any treatment-related gross or pathology findings were observed at any dose in animals designated for the reproductive evaluation.

3.4. Developmental toxicity

There were no NaPFHx-related deaths or gross postmortem findings in dams at any dose. Maternal toxicity occurred at 500 mg/(kg day) and consisted of reductions in body weight parameters of total weight gain from GD 6 to 21 and overall net gain [body weight gain (GD 6–21) minus products of conception on day 21], which were 19% and 26% lower than control group, respectively (Table 8) and a 5% reduction in food consumption (data not shown). Developmental toxicity was limited to a ~10% decrease in fetal weight at 500 mg/(kg day) (Table 8).

3.5. Genotoxicity

No toxicity, NaPFHx precipitation, or positive mutagenic responses were observed at any dose level or with any tester strain in either the presence or the absence of S9 metabolic activation in the Bacterial Reverse Mutation (Ames) Test (Supplemental Table S1 and S2). Substantial toxicity, defined as a reduction in the mitotic index of >50% in the NaPFHx treated cell culture as compared with the mitotic index in the concurrent vehicle control, was observed in the *in vitro* mammalian chromosome aberration test in all testing conditions. A 69% reduction was observed at 3860 μg/mL in the 4-h non-activated test condition, and 57% and 74% reductions at 1000 μg/mL in the 4-h activated and 22-h non-activated test conditions, respectively. The percentage of cells with structural or numerical aberrations in the NaPFHx-treated groups was not significantly increased above that of the vehicle control at any concentration (Supplemental Table S3). NaPFHx was not found to induce structural or numerical chromosome aberrations in human peripheral blood lymphocytes in either the non-activated or S9-activated test systems.

4. Discussion

The results of these studies indicate that the NOAEL for NaPFHx in male rats is 20 mg/(kg day), based on nasal lesions following approximately 90 days of exposure by oral gavage and weight loss in P1 rats in a reproductive toxicity study following exposure to 100 or 500 mg NaPFHx/(kg day). There were no changes in clinical chemistry parameters indicative of NaPFHx-related organ toxicity. Decreases in red blood cell mass parameters in males and females at 500 mg/(kg day) were observed and considered adverse. The erythroid regenerative response manifest in peripheral blood correlated with histopathology findings of increased splenic extramedullary hematopoiesis and/or bone marrow erythroid hyperplasia for a number of high-dose animals. Other effects on hematology were considered non-adverse, due to their minimal effect or absence of a histopathological correlate. The increases in hepatic β-oxidation at 500 mg/(kg day) in both sexes and at 100 mg/(kg day) in males at the end of dosing were consistent with secondary responses to the receptor-mediated upregulation of metabolic enzyme production and were not considered adverse.

The NOAEL for nasal lesions was 20 mg/(kg day) based on olfactory epithelium degeneration and atrophy observed in 4 of 10 male and 5 of 10 female rats dosed with 100 mg/(kg day) NaPFHx. Benchmark dose (BMD) analysis determined a 10% increase in risk (BMD10) of 30 mg/(kg day) for male rats and 13 mg/(kg day) for the

95% confidence limit (BMDL10) using a log probit fit of the data. For female rats the corresponding values were 45 and 21 mg/(kg day), respectively, for the BMD10 and the BMDL10, using a log logistic analysis. Degeneration of the olfactory epithelium has been reported in the rodent nose after inhalation exposure to a number of different irritating chemicals (Hurtt et al., 1988; Keenan et al., 1990; Lee et al., 1992; Newton et al., 2002). When chemicals are administered by routes other than inhalation, respiratory tissues can be exposed to a chemical or its metabolites, such as reports of olfactory epithelial lesions following intraperitoneal or gavage dosing (Genter et al., 1992; Larson et al., 1995; Bergman et al., 2002). In rodents, olfactory epithelium has a high level of turnover (Moulton, 1974) and a considerable ability to biotransform chemicals (Dahl and Hadley, 1991; Deamer et al., 1995; Genter, 2004). Biotransformation enzymes are also present in human nasal tissue (Zhang et al., 2005) but at lower levels (Feng et al., 1990; Bogdanffy et al., 1998; Heydens et al., 1999). For example, Feng et al. (1990) reported cytochrome P-450 activity (7-ethoxy coumarin-O-deethylase) in human nasal tissue was 0.25% of the same activity in rats.

It is unlikely, however, that metabolism of NaPFHx explains the degeneration/atrophy and other nasal lesions observed in rats dosed with 100 or 500 mg/(kg day), since no loss of parent compound was observed after 2 h of incubation with rat hepatocytes (unpublished data). Other possible mechanisms for nasal lesions following noninhalation routes of exposure suggested by Sells et al. (2007) include inhalation of volatiles from the stomach after oral dosing or inhalation of exhaled parent or metabolite. Given the nonvolatile nature of NaPFHx, these mechanisms are probably not applicable.

Considering the surfactant properties of NaPFHx, the nasal tissue in rats may be sensitive to the irritating properties reported for materials in this class of chemicals. Lieder et al. (2009) reported multifocal necrosis of olfactory epithelium in a few of the Sprague–Dawley rats dosed with 600 mg/(kg day) potassium PFBS and attributed those results to the strong surfactant properties of that material. In an attempt to put rat nasal cytotoxins into perspective, Jeffrey et al. (2006) examined 14 chemicals that caused nasal damage in rodents but were not carcinogens in rodents. None of these chemicals has been identified as a systemic nasal toxicant in humans and the authors concluded that cytotoxic lesions found solely in rodent nasal mucosa do not necessarily predict toxicity in those tissue areas in humans. It appears likely, based upon the NOAEL of 20 mg/(kg day) and very low levels of potential human exposure, that NaPFHx would not have a deleterious effect on nasal tissue in humans.

Increased liver weights and microscopic hepatocellular hypertrophy were present in males and females at 500 mg/(kg day) and in males at 100 mg/(kg day). Liver weight effects in the 500 mg/(kg day) groups showed some, but not complete recovery after 30- and 90-day treatment-free periods. Microscopic hepatocellular hypertrophy correlated with increases in liver weights and was observed at the same doses which resulted in peroxisomal proliferation, as indicated by increases in β-oxidation activity (Fig. 3). These liver effects were consistent with secondary responses to the receptor-mediated upregulation of metabolic enzymes and were not considered adverse. Exposure to xenobiotics commonly induces hepatic metabolizing enzymes in laboratory animals (Paynter et al., 1985; Greaves, 1990; Sipes and Gandolfi, 1991).

The relative kidney weight increases observed at 500 mg/(kg day) were not associated with microscopic or clinical pathology changes indicative of renal toxicity. Therefore, these changes were considered to be non-adverse and, as in the liver, were likely secondary to the receptor-mediated upregulation of metabolic enzymes.

Hypertrophy of thyroid follicular epithelium was present in male and female rats, primarily in the 500 mg/(kg day) groups, including the recovery groups. Therefore, increased thyroid weights in 500 mg/(kg day) females after 30 days of recovery may be treatment-related. Thyroid changes were minimal and likely secondary to receptor-mediated upregulation of metabolic enzymes in the liver. Thyroid hypertrophy is a common finding in rats in association with induction of hepatic microsomal enzymes, and in this study was seen only at doses that also produce liver hypertrophy. Increased activity of the enzyme UDP-glucuronyltransferase, as part of a spectrum of induced cytochrome P450 isoenzymes, leads to increased biliary excretion of the thyroid hormone, T4. This excretion results in elevation of thyroid stimulating hormone (TSH), which produces hypertrophy of follicular epithelial cells. Due to the species-specific short half-life for T4 in rodents, rats are uniquely sensitive to thyroid hormone perturbation in association with induction of liver enzymes (Capen, 1997). Consequently, the thyroid follicular cell hypertrophy observed in the current study was considered potentially adverse, although it is likely that this response is not relevant to nonrodent species (Alison et al., 1994).

Statistically significant but minimal increases in mean urine fluoride were present in the 500 mg/(kg day) male and female groups at day 92 and 93, respectively. However, the mean values in these groups, although statistically increased relative to their time-matched controls, were similar to or less than the mean value of the control groups at the subsequent time points. A statistically significant increase in urinary fluoride was also present in the 500 mg/(kg day) female group following 30 days of recovery. Based on the *in vitro* metabolism studies mentioned above, NaPFHx is metabolically stable and does not release free fluoride. Consistent with these results is the observation that there were no increases in plasma fluoride in the current study nor evidence of any broken teeth or degeneration of enamel organ ameloblast cells, both of which have been reported in rats after a 3-month exposure to a fluoroalkylethanol mixture that resulted in elevated plasma and urine fluoride levels (Ladics et al., 2005). Therefore, the slight increases in urine fluoride in some 500 mg/(kg day) groups were likely spurious or related to individual animal variation in urine flow and/or urine pH, factors known to influence normal fluoride elimination.

The results reported here are complementary to those presented by Chengalis et al. (2009a). In a subchronic oral gavage study of PFHxA, rats were dosed with 10, 50, or 200 mg/(kg day) for 90 days compared to 20, 100, and 500 mg/kg NaPFHx/day in the current study. PFHxA affected body weight gains at all doses, but not in a dose-responsive manner, so no NOEL could be set based on the data. Statistically significant decreases in body weight were reported for males at 50 and 200 mg/(kg day), compared to 500 mg/(kg day) in the present study. Decreases in serum cholesterol and calcium were reported at 50 and 200 mg/(kg day) in male rats and lower globulin levels in male and female rats at 200 mg/(kg day). Decreases in these same parameters were observed in male rats in the currently reported study at 100 mg/(kg day) (cholesterol) and 500 mg/(kg day) (calcium). No effects were observed on globulin levels in female rats at any dose, although decreases were seen in male rats at 100 and 500 mg/(kg day). None of these clinical chemistry changes were interpreted as adverse. NOAELs were reported as 50 and 200 mg/(kg day) for male and female rats, respectively, although the authors stated that the slight increases in liver weight and hepatocellular hypertrophy seen at 200 mg/(kg day) in male rats were consistent with an adaptive response following treatment with peroxisome proliferators, as reflected in the increase in β-oxidation activity observed at this dose. Similar dose responsive increases in liver weight and hepatocellular hypertrophy were seen in the current study, in that values or incidences at 100 and 500 mg/kg NaPFHx/day bracket the data presented for 200 mg/kg PFHxA/day in the Chengalis et al. (2009a) study. Effects on red blood cell parameters were also noted at the high dose in male and female rats in each study, as well as in studies with PFBS

(Lieder et al., 2009) and PFHxS (Butenhoff et al., 2009). The non-adverse compound-related increases in liver and kidney weights observed at 100 and 500 mg/kg NaPFHx/day were also observed at 200 mg/kg PFHxA/day (liver) and 50 mg/kg PFHxA/day (kidney; female only), respectively. It is not clear why the mild to minimal treatment-related nasal lesions seen at 100 and 500 mg/(kg day) were not reported in the Chengalis et al. (2009a) study, given the identical rat strain used and the consistent similarity of the other effects.

It is of interest to compare results from this study of NaPFHx to results reported for two homologous perfluorocarboxylic acids (PFCAs): perfluorooctanoate [PFO, $F(CF_2)_7CO_2^-$, 8-carbons] and perfluorobutanoate [PFB, $F(CF_2)_3CO_2^-$, 4-carbons], and perfluorohexanesulfonate [PFHxS, $F(CF_2)_6SO_3^-$], a six-fluorinated carbon perfluoroalkyl sulfonate (PFAS). In the current study relative liver weights were 160% of controls at 500 mg/(kg day), with a BMDL10 of 59 and 191 mg/(kg day) for male and female rats, respectively, dosed with NaPFHx. The NOEL for liver hypertrophy was 20 mg/(kg day) for NaPFHx, whereas the LOAEL for effects on red blood cell parameters was 500 mg/(kg day). In comparison, relative liver weights were 156% of control at 6.5 mg/(kg day) in rats (Perkins et al., 2004) following 90 days of dietary ammonium perfluorooctanoate (APFO), with a BMDL10 of 0.44–0.72 mg/(kg day) for effects on relative liver weight (Goeden et al., 2008). The NOEL for liver hypertrophy was 0.06 mg/(kg day) (1 ppm) for APFO (Perkins et al., 2004). Further, a LOAEL of 30 mg PFBA/(kg day) for 90 days in rats was reported to cause a 23% increase in liver weights and ~5% decrease in red blood cell parameters (J. Butenhoff, personal communication, 2009), compared to a LOEL of 100 mg/(kg day) for liver effects and a LOAEL of 500 mg/(kg day) for effects on red blood cell parameters reported herein. Finally, in male rats dosed by gavage with potassium PFHxS in a modified OECD 422 guideline study, the NOAEL for liver and thyroid hypertrophy was reported to be 1 mg/(kg day) (Butenhoff et al., 2009).

Developmental and reproductive comparisons can also be made among perfluorinated acids. In the current study, decreases in maternal and fetal weights were observed following 500 mg/(kg day) NaPFHx, but no compound-related effects were observed at any dose on the incidence of fetal, visceral, or skeletal variations. Reproductive effects were limited to decreases in maternal body weight gains and F1 pup weights at 500 mg/(kg day); no effects were observed at any dose on any reproductive indices. For PFOA, no reproductive end points were affected in rats exposed to APFO in a 2-generation reproduction study with doses ranging from 1 to 30 mg/(kg day) (Butenhoff et al., 2004). In CD-1 mice, however, effects on neonatal survival were noted following exposure throughout gestation to 5–40 mg/kg PFOA/day, with subsequent growth impairment and developmental delays in survivors (Lau et al., 2006). Neonatal survival appears to depend upon the presence of PFOA-activated peroxisomal proliferation-activated receptor-α (PPAR-α), since 129S1/SvlmJ PPAR-α knockout litters exposed to 1 mg PFOA/kg on gestation days 1–17 had 96% of pups alive on postnatal day 22, compared to 43% pup survival in wild type litters (Abbott et al., 2007).

In CD-1 mice dosed by gavage with 35, 175, or 350 mg/(kg day) PFBA, significant delays in eye-opening in offspring were noted in all dose groups, accompanied by slight delays in puberty onset in the two higher doses (Das et al., 2008). PFBA exposure during pregnancy did not result in decreased neonatal survival or delays in postnatal growth in CD-1 mice. Finally, when one compares the effects on reproductive outcome between the C6 and C8 sulfonated molecules, PFHxS and PFOS, no reproductive or developmental effects were observed in rats exposed to 0.2, 1, 3, or 10 mg/(kg day) PFHxS (Butenhoff et al., 2009). In contrast, in studies where animals were exposed to PFOS during gestation, the BMDL5 for postnatal survival to day 8 in rats was 0.6 mg/(kg day) compared to 3.9 mg/(kg day) for postnatal survival to day 6 in mice dosed with PFOS (Lau et al., 2003). In a study more similar in design to the current one, female rats were dosed with PFOS for 42 days prior to mating and continued through gestation, resulting in a BMDL5 of 0.9 mg/(kg day) for postnatal survival of pups to lactational day 5 (Luebker et al., 2005).

Unlike PFHxS, PFOS and PFOA, and like PFBA, PFHxA is rapidly eliminated, which may contribute to its lack of effect on developmental and reproductive parameters. Carbon-14-labelled PFHxA has been reported to be almost completely absorbed, not metabolized, and nearly 100% excreted in the urine within 24 h in male and female CD-1 mice and CD rats (Gannon et al., 2009). Similarly, in male and female CD rats dosed daily with 50–300 mg/(kg day) of PFHxA for 25 days, the half-life for urinary elimination was 2–3 h (Chengalis et al., 2009b).

In conclusion, administration of NaPFHx by oral gavage for approximately 90 days was associated with pathology findings in male and female rats at 100 and 500 mg/(kg day). Other adverse changes observed at 500 mg/(kg day) included hematology and thyroid pathology. No NaPFHx-related anatomic pathology, hepatic peroxisomal β-oxidation, neurobehavioral, or clinical pathology changes were present in male or female rats administered 20 mg/(kg day), resulting in a NOAEL for subchronic toxicity of 20 mg/(kg day). The maternal and developmental NOAEL for developmental toxicity was 100 mg/(kg day). For the reproductive portion of the 90-day subchronic study, the P_1 adult rat NOAEL was 20 mg/(kg day), based on reduced body weight parameters, whereas the NOAEL for reproductive toxicity was 100 mg/(kg day), based on reduced F_1 pup weights. Finally, NaPFHx was concluded not to be genotoxic, with negative results in both the bacterial reverse mutation assay and *in vitro* chromosomal aberration assay.

Conflict of interest statement

All authors are employed by the DuPont Company.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2009.07.011.

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